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14. ABSTRACT
The initial objective of this proposal is to characterize the regulation of BHC80 on the Snail-LSD1 interaction, and explore the prognostic value of BHC80 as biomarker for the metastasis of breast cancer. In the first year of the study, we aim to delineate the mechanisms underlying the interplay among BHC80, LSD1 and Snail. More specifically, we plan to verify if 1) the PHD finger of BHC80 interacts with the SNAG domain of Snail; 2) BHC80 affects the protein stability of Snail and LSD1; and 3) BHC80 affects LSD1/Snail binding to E-cadherin promoter. After we over-expressed the wild type and PHD finger deletion mutant of BHC80 in HEK293 cells and performed co-immunoprecipitation experiments, we found there was no significant difference between wild type and mutant BHC80 in regard to Snail binding affinity. We further identified that in addition to BHC80, PARP1 was a component of Snail/LSD1 complex. According to our initial data, BHC80 might cooperate with PARP1 to affect the protein stability of Snail and LSD1, as well as Snail/LSD1 binding to the target gene promoter. The identification and functional characterization of PAPR1 will potentially help us better understand the role of the Snail/LSD1 complex in breast cancer metastasis, and explore the prognostic value of this complex as a biomarker.

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Introduction

The initial objective of this proposal is to characterize the regulation of BHC80 on the Snail-LSD1 interaction, and explore the prognostic value of BHC80 as a biomarker for the metastasis of breast cancer. The metastatic cascade consists of four distinct steps: 1) invasion, 2) intravasation, 3) extravasation, and 4) metastatic colonization. Epithelial-mesenchymal transition (EMT), with the hallmark being loss of E-cadherin expression, is a critical mechanism governing the invasion process. Snail is a master regulator of EMT through transcriptional repression of E-cadherin. In our recent study, we identified Snail, LSD1, BHC80 form a protein complex. While we have demonstrated that LSD1 enhances the protein stability of Snail and cooperates with Snail to induce EMT, the function of BHC80, as well as potential other protein components is poorly understood. According to our preliminary data, BHC80 negatively regulates the interaction of Snail with LSD1. We planned to further characterize the mechanism on how BHC80 contributes to EMT and breast cancer metastasis.

Body

The Statement of Work indicated two studies to be accomplished in the first year of the fellowship. We have substantially completed these studies, during which we also had significant new findings. While we found part of our results deviated from our initial hypothesis, the majority of the data came out as expected. We also identified a new protein component of the Snail/LSD1 complex, and have performed initial experiments to characterize the function of this protein, which pretty much addressed the unexpected part of our results. More specifically, after we constructed the wild type and PHD finger deletion mutant of BHC80 and performed co-immunoprecipitation experiments, we found there was no significant difference between the wild type and mutant BHC80 in regard to Snail binding affinity. However we identified that in addition to BHC80, PARP1 was a component of Snail/LSD1 complex. According to our initial data, BHC80 might cooperate with PARP1 to affect the protein stability of Snail and LSD1, as well as Snail/LSD1 binding to the target gene promoter.

In the following we briefly list the original SOW and our accomplishments:

SOW – Study 1: Can the PHD finger of BHC80 interact with the SNAG domain of Snail?

We have successfully performed endogenous co-immunoprecipitation experiments to examine the Snail-BHC80 interaction in MDA-MB-231, SKBR3 and BT549 breast cancer cells, which have high levels of Snail expression. As shown in Fig.1, Snail-BHC80 interaction was confirmed in all of the three cell lines. Secondly, we have successfully constructed wild-type and PHD finger deletion mutant of BHC80 using the pCMV-Tag Epitope Tagging Mammalian Expression System (Stratagene) available in our lab. We co-transfected HEK293 cells with HA-tagged Snail and Flag-tagged wild-type/mutant BHC80, and then performed exogenous co-immunoprecipitation experiments. We found there was no significant change in regard to Snail binding affinity when the PHD domain of BHC80 was deleted. We reasoned that in addition to

the PHD finger, other domain(s) of BHC80 also contributes to Snail binding. Since the PHD domain along was not sufficient for Snail binding, we did not continue to perform systematic site-directed mutagenesis analysis as planned in the original SOW. We are currently communicating with Dr. Young-In Chi on the computer modeling analysis to confirm our co-immunoprecipitation results. In addition to the results mentioned above, we have identified new protein component of the Snail/LSD1 complex: PARP1 (Poly [ADP-ribose] polymerase 1) (Fig. 2). PARP1, also known as NAD^+ ADP-ribosyltransferase 1, is an enzyme that modifies nuclear proteins by poly ADP-ribosylation. In comparison to BRCA, which repairs DNA double strand breaks, PARP family proteins act on single strand breaks. It has been demonstrated that PARP1 is involved in cell differentiation, proliferation and tumor transformation. The identification of PARP1 as a component of the Snail/LSD1 complex indicates that PARP1 might cooperate with BHC80 in regulating protein stability as well as target gene promoter binding. Therefore in the following studies we also incorporated PARP1 as we were planning our experiments.

SOW – Study 2: Can BHC80 affect the protein stability of Snail and LSD1?

Firstly, we used siRNA to knockdown the expression of BHC80 in MDA-MB-231, SKBR3 and BT549 cells, and examined the protein levels of Snail and LSD1 by western blot. There were only slight changes in regard to protein levels.

Secondly, as we found PARP1 appeared as a critical component of the Snail/LSD1 complex, we planned to further characterize the function of PARP1. We tried to figure out if PARP1 is involved in the association of Snail with LSD1. As shown in Fig. 3A, both PARP1 over-expression and doxorubicin treatment enhanced Snail-LSD1 binding in HEK293 cells. As for MDA-MB-157 cells, while doxorubicin consistently had positive effect, either treatment of PARP1 inhibitor AZD2281 or PARP1 knockdown significantly reduced Snail-LSD1 affinity (Fig. 3B). These results indicated that PAPR1 plays a regulatory role in Snail-LSD1 interaction.

Thirdly, we tried to investigate whether Snail can either undergo poly(ADP-ribosyl)ation or simply interact with PARP1 through potential pADPr binding motif, as established by previous studies. Through sequence alignment we identified three highly conserved residues Arg151, Lys152 and Ala153 of Snail protein to be in concert with the corresponding residues of the pADPr binding motif, in which the positively charged lysine and arginine are strictly followed by alanine, isoleucine, leucine or valine (Fig. 4A). While the surrounding sequence does not exactly follow the rule as refined by Gagne and colleagues, the presence of the most essential residues still indicates the potential pADPr docking site on Snail protein. To test this hypothesis, we generated Snail point mutant R151A/K152A and examined its interaction with PARP1. As shown in Figure 4B, the mutant significantly lost PARP1 binding affinity compared to wild-type Snail, indicating that R151, K152 are critical for PARP1 association. Interestingly, the Snail mutant also significantly lost the binding affinity for LSD1 with or without treatment of Doxorubicin (Figure 4C). When the cells were treated with Gallotannin, an inhibitor of poly(ADP-ribose) glycohydrolase (PARG) which catalyzes the degradation of pADPr, the association of Snail-LSD1 was enhanced (Figure 4D). To investigate whether Snail can also undergo poly(ADP-ribosyl)ation, we immunoprecipitated Snail protein from abovementioned stable HEK293 cells, as well as MDA-MB-157 and HCT116 cells, and performed western-blot

using antibody against pADPr . As shown in Figure 4E, Snail protein was poly(ADP-ribosyl)ated, with the effect enhanced by Doxorubicin and suppressed by AZD2281. There was no significant difference in regard to the level of poly(ADP-ribosyl)ation on wild-type and the mutant Snail (data not shown), suggesting the potential existence of multiple modification sites. Furthermore, the Snail mutant became less stable compared to the wild-type (Figure 4F), which was in accord with our previous finding that formation of Snail-LSD1 complex was required for maintaining the stability of each component. Together, we demonstrated that PARP1 interacts with a potential pADPr binding motif of Snail and mediates Snail-LSD1 association as well as protein stability, and Snail protein is subject to poly(ADP-ribosyl)ation on multiple residues.

SOW – Study 3: Can BHC80 affect Snail-LSD1 complex activity on E-cadherin promoter suppression?

According to SOW, this study would be carried out during months 11-18. We planned to first perform chromatin immunoprecipitation (ChIP) experiments to examine the interaction of LSD1/Snail with E-cadherin promoter in MDA-MB-231, SKBR3 and BT549 breast cancer cells with or without knockdown of BHC80 and PAPR1 expression. These experiments are currently underway as we are optimizing experimental condition in different cell lines.

Key research accomplishments

1 Demonstrate that in addition to the PHD finger, other domains of BHC80 also contribute to Snail binding.
2 Find new protein component of the Snail/LSD1 complex: PARP1.
3 Demonstrate that PARP1 cooperates with BHC80 in mediating Snail stability as well as Snail/LSD1 binding to E-cadherin promoter.
4 Find the PARP1 binding site of Snail protein.
5 Demonstrate that Snail can be parylated by PAPR1.
6 Demonstrate that while BHC80 decreased Snail-LSD1 interaction, PARP1 can enhance protein binding.

Reportable outcomes

To date, we are preparing a manuscript for journal submission. While we have already gained significant data as shown above, still more experiments would be needed.

Conclusion

In the first year of this fellowship we have substantially completed the proposed studies, including verification of the role of the PHD finger in mediating BHC80-Snail interaction, and demonstration of the role of BHC80 in mediating the protein stability of Snail. The functional study of BHC80 in mediating Snail-LSD1 binding to E-cadherin promoter is currently in progress.

A mild disappointment is that we haven’t narrowed down the protein domain responsible for Snail-binding to the originally expected PHD finger. We found that in addition to the PHD

finger, other domain(s) of BHC80 also contributes to Snail binding. By contrast, we have achieved a more significant accomplishment: we found a new protein component of Snail/LSD1 complex in the name of PAPR1. We have demonstrated that PARP1 can parylate Snail, and mediate Snail-LSD1 interaction. Furthermore, our initial studies indicated that PARP1 might cooperate with BHC80 to mediate the Snail/LSD1 binding to the target gene promoter.

The identification of PAPR1 as a new protein component has significant implications and directs our study to a more thorough and precise way. Functional characterization of both PAPR1 and BHC80 will give us a better understanding of the role of the Snail/LSD1 complex in breast cancer metastasis. In the following years we will continue to follow the proposal as outlined in the SOW to study the function of BHC80; in addition, we will put PARP1 into consideration and evaluate its potential cooperation with BHC80 in breast cancer metastasis.

References

N/A

Appendices

N/A

__Supporting Data__

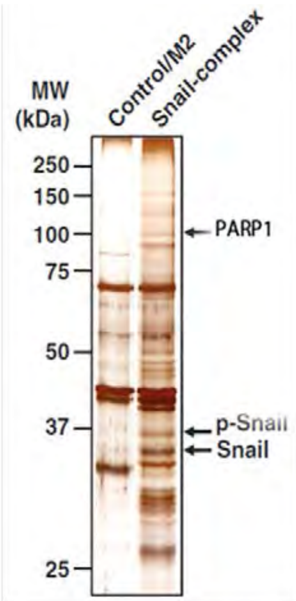

Figure 1 Mass-spectrometry analysis to identify PAPR1 as an interacting protein of Snail.

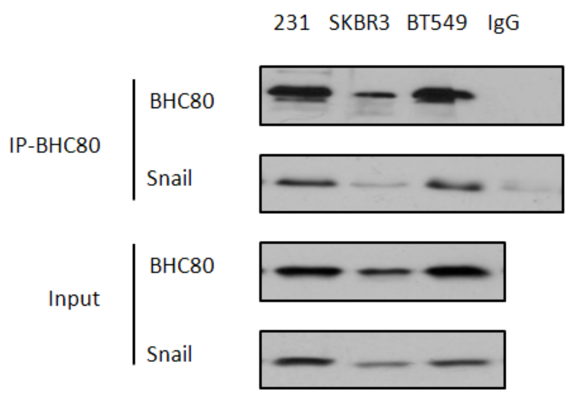

Figure 2 Snail interacts with BHC80 in breast cancer cell lines MDA-MB-231, SKBR3 and BT549.

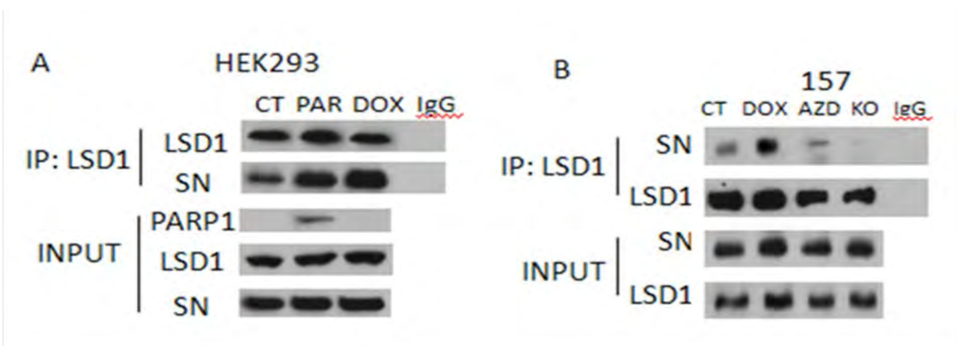

Figure 3 PAPR1 mediated Snail-LSD1 interaction. A) HEK293 cells with Snail/LSD1 overexpression were either co-transfected with PARP1 or treated with Doxorubicin. The interaction of Snail with LSD1 were examined by Western blot. B) MDA-MB-157 cells were either treated with Doxorubicin, or PARP1 inhibitor, or transfected with PAPR1 siRNA. The interaction of Snail with LSD1 were examined by Western blot.

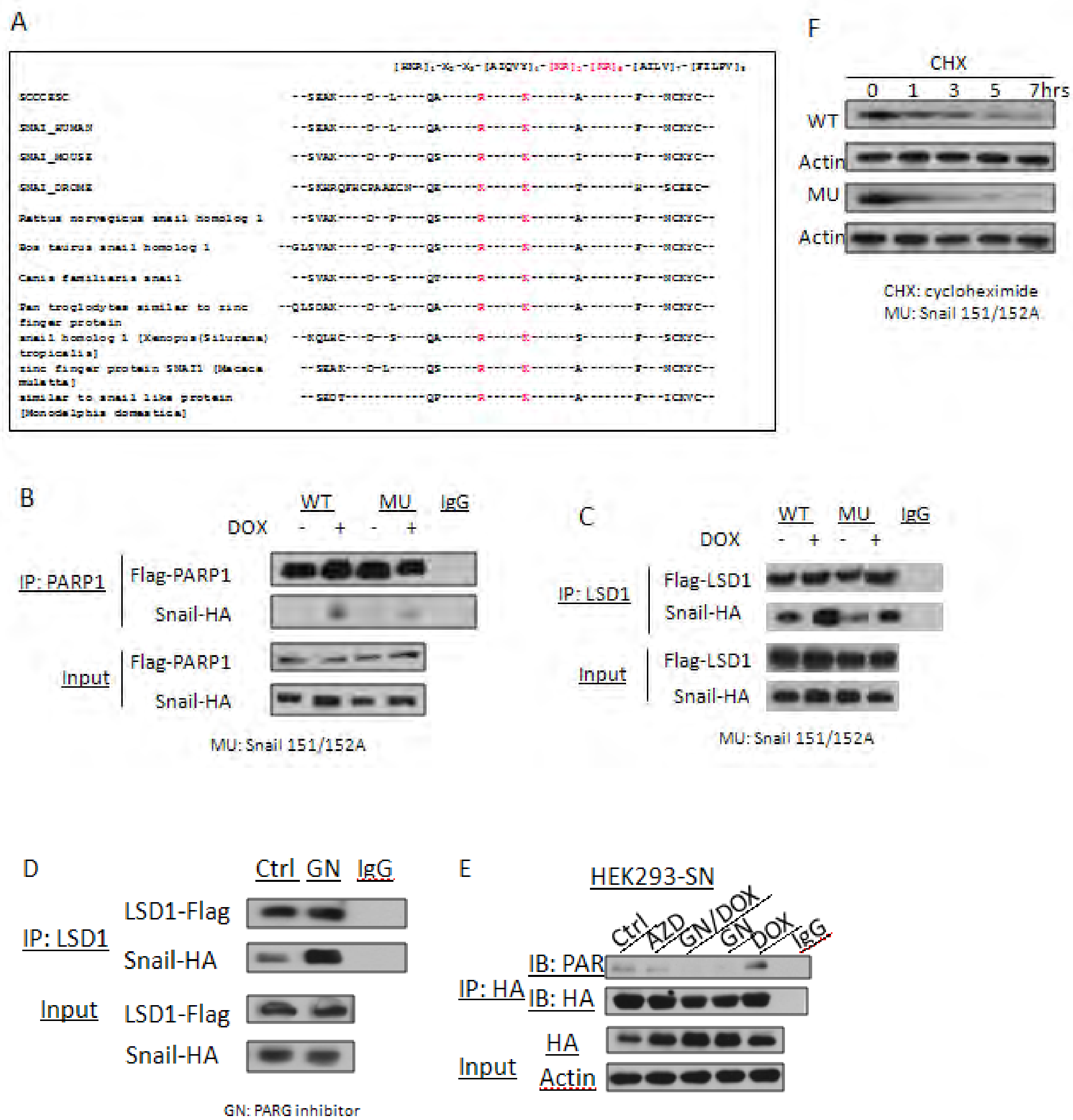

Figure 4 Snail is parylated by PAPR1. A) Sequence alignment of Snail proteins from different species. The conserve R and K are shown in red. B) HEK293 cells were transfected with PAPR1 and wild type or mutant Snail. Immunoprecipitation experiments were performed and Snail and PARP1 were detected by Western blot. C) HEK293 cells were transfected with LSD1 and wild type or mutant Snail. Immunoprecipitation experiments were performed and Snail and LSD1 were detected by Western blot. D) HEK293 cells were co-transfected with Snail and LSD1. Cells were treated with Gallotannin and the interaction of Snail with LSD1 was examined. E) HEK293 cells stably expressing Snail were treated with AZD2281, Doxorubicin, or Gallotannin, Snail protein was immunoprecipitated and protein parylation level was examined. F) HEK293 cells were transfected with wild type or mutant Snail. Cells were then treated with Cycloheximide and the protein degradation was examined by Western blot.